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CHAPTER 35

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Concentration and Accumulation of Metallic Ions by the Bacterium *Zoogloea*

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Zoogloea-producing bacteria are common in natural waters and appear to be able to survive and grow in the presence of rather high concentrations of contaminants. They have been isolated consistently from oxidative waste treatment systems where they have been reported to cause a rapid decrease in the biochemical oxygen demand.

Our interest in these organisms is centered upon the unique properties of the zoogleal material surrounding the cells. It is lacking in nonzoogloea-forming organisms.

Studies have been made of several of these bacteria and their ability to concentrate several metallic ions.

Biological waste treatment processes convert reduced organic substances to microbial cell mass. The energy required to drive this synthetic process is derived from oxidative reactions catalyzed by the enzyme systems of the microorganisms. Once the organic material has been converted to microbial cells, biological flocculation becomes an essential part of the process, forming the basis for removal of partially oxidized material from the water. Relatively little is known about the role of dissolved minerals in the treatment process, particularly in relation to growth of microorganisms. Current opinion is that mineral content as well as organic content of treated waste plays an important role in stimulating growth of algae and other plant forms in receiving waters. (Eyster, 1964; Fogg, 1965).

There are several types of floc-forming bacteria indigenous to natural or contaminated water. Some of the floc formers have a characteristic growth habit described as a gelatinous or zoogleal matrix in which cells are embedded.

Gram-negative, single polar flagellated rods of this type comprise the genus *Zoogloea*. This genus is differentiated from members of the genus *Pseudomonas* by its characteristic zoogleal matrix. The specific reactions of the organisms which have taxonomic value have been described elsewhere (Dugan and Lundgren, 1960; Crabtree and McCoy, 1967; Bergey's Manual, 1957). Our interest in *Zoogloea* species concerns the ability of the organism to concentrate and accumulate dissolved chemicals from solution. We are also attempting to elucidate the functional role of the gelatinous matrix in the metabolic activities of the organism. This study is stimulated by potential application in the following areas: 1) as a means for removal of minerals in tertiary waste treatment processes resulting in a retarding influence on the eutrophication process in receiving waters, 2) recovery of isotope contaminants from aquatic environments, and 3) basic information concerning nutritional requirements and environmental influences on activity of the organisms will allow more efficient design of specialized aerobic treatment facilities.

The metal ion uptake of the gelatinous matrix producing *Zoogloea ramigera* strain 115 (Friedman and Dugan, 1967, *unpublished results*) and of the non-gelatinous matrix

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producing strain 1-16-M of Crabtree et al. (1966) were studied. The gelatinous or zoogleal matrix which surrounds the 115 cells is shown in Figure 1, a photomicrograph of cells cultivated in the defined arginine medium of Crabtree et al. and stained with 1% aqueous crystal violet. Figure 2 shows 1-16-M cultivated and stained in the same manner; no extracellular gelatinous material can be seen.

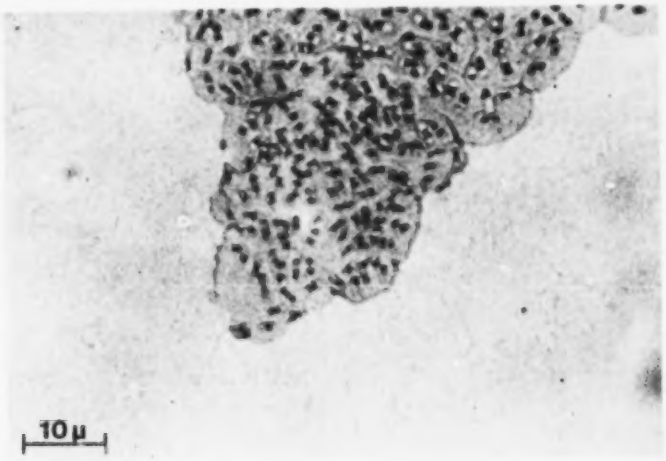

FIG. 1. Photomicrograph of *Zoogloea* 115 floc, stained with 1% aqueous crystal violet, showing cells embedded in the zoogleal matrix.

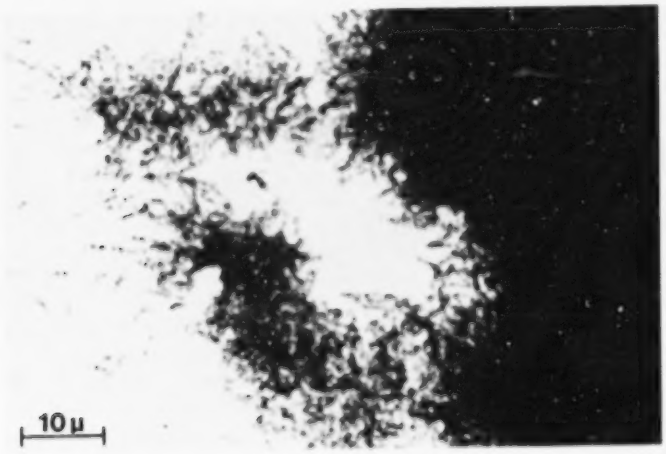

FIG. 2. Photomicrograph of *Zoogloea* 1-16-M floc, stained with 1% aqueous crystal violet showing lack of zoogleal matrix. Cells were cultured in an identical manner to those shown in Figure 1.

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Gross Uptake of Metallic Ions

Gross uptake of several colored metal ions by the cells was used as a screening procedure. Colored metal ions were selected because we could obtain a visual indication of metal accumulation by the color of the cell pellet after centrifugation. The following procedure was used: 0.1 ml of a standardized pre-washed cell suspension in 0.3 M phosphate buffer was inoculated into a series of 250-ml Erlenmeyer flasks, each containing 50 ml of trypticase soy broth (TSB, Baltimore Biological Laboratories). The flasks were incubated at 28 C for 48 hours on a reciprocal shaker (100 oscillations/min). The cells from each flask were centrifuged and washed twice using 10 ml phosphate buffer and then resuspended in 50 ml of sterile distilled water in 250-ml flasks. One ml each of stock solutions of $CoCl_2$, $CuCl_2$, $FeCl_3$, and $NiCl_2$ were added separately to give the final concentrations of metal ion per flask shown in Table 1. The cell-metal ion suspension was shaken at 28 C for 18 hours on a reciprocal shaker and the cells were then separated from the solution by centrifugation. Accumulation of colored ions in the cell paste at the bottom of the tubes was readily seen. Metal ion concentration in the supernatant was determined via atomic absorption spectrometry (Perkin-Elmer model) and compared to initial concentrations. Results of these determinations are listed in Table 1. These experiments gave us an indication of the extent to which pregrown cells would concentrate and accumulate metal ions in a nongrowth situation. The 115 cells have a remarkably high affinity for Co^{++}, Cu^{++}, and Fe^{+3}, and somewhat less for Ni^{++}. Values obtained after contact periods of 2 and 3 days indicate that no further uptake of ions occurs beyond 18 hours. No data were obtained for time intervals less than 18 hours in this experiment. Comparison of data obtained using 115 cells (zoogleal matrix) with data from I-16-M cells (no zoogleal matrix) shows that approximately twice as much metal ion is accumulated by the zoogleal strain. This suggests that about half the ion uptake is concentrated in the zoogleal matrix surrounding the cells, although no direct evidence to support this is yet available. Uptake of metal ions by I-16-M cells indicates that non-zoogleal cells can concentrate a considerable amount of metal ion. Total uptake therefore, seems to be due to combined influence of cell and surrounding matrix.

The cells accumulate 34% of their total weight as copper ions and approximately 25% of their weight as cobalt ions. This precludes use of this type of experiment to determine the time course of metal uptake by growing cells. No accurate cell weight determinations can be made in the presence of relatively high concentrations of ions. Cell counting techniques are not feasible with the cells embedded in a zoogleal matrix. More precise data on metal uptake by both growing and nongrowing cells were obtained using the radioactive element $Zn^{65}Cl_2$.

Uptake of Zn^{65}

Accumulation of Zn^{65} by actively-growing cells was determined in the following manner: 0.1 ml of a standardized pregrown washed cell suspension was inoculated into 50 ml of TSB in 250-ml Erlenmeyer flasks. Zn^{65} was added to the flasks in one of 3 ways: 1) Zn^{65} to give a final concentration of 1 μC in 50 ml growth medium (Sp.Act. 1.11 mC/mg), 2) Zn^{65}, 1 μC, plus 50 μg carrier Zn as $ZnCl_2$ in 50 ml growth medium, 3) Zn^{65}, 1 μC, plus 500 μg Zn carrier. The cells were then incubated at 28 C for periods up to 14 days on a reciprocal shaker. Flasks containing the culture were removed from the shaker at specified intervals (Fig. 3, 4) and centrifuged. Five ml aliquots of supernatant were removed and counted. The cell paste was resuspended

TABLE 1. *Values showing initial concentration of metallic ion in solution (μg/ml) and concentration after shaking for 18 hours in presence of* Zoogloea *115 (0.1 g, dry wt) or* Zoogloea *I-16-M (0.12 g, dry wt)*

	Cobalt as $CoCl_2 \cdot 6H_2O$			Copper as $CuCl_2 \cdot 2H_2O$			Iron as $FeCl_3 \cdot 6H_2O$			Nickel as $NiCl_2 \cdot 6H_2O$		
Initial Concentration												
Zoogloea 115	50	240	500	90	400	800	34	180	350	50	250	500
Concentration after 18 hours	1.3	2.2	3.5	73	97	121	0.5	3.2	4.3	41	190	250
% removal	97.5	99.1	99.3	18.9	75.8	84.9	98.5	98.2	98.8	18	24	50
Total removed, mg/100 mg cells (dry wt)	2.4	11.9	24.8	0.8	15.1	34	16.7	8.8	17.3	0.4	3	12.5
Zoogloea I-16-M												
Concentration after 18 hours	2.8	64	310	4.7	150	420	0.3	1.1	187	32.6	164	376
% removal	94.5	73.3	38	94.8	62.5	47.5	99.1	99.4	46.6	34.8	34.4	24.8
Total removed, mg/100 mg cells (dry wt)	2.3	8.8	9.5	4.3	12.5	19	16.8	9	8.2	0.9	4.3	6.2

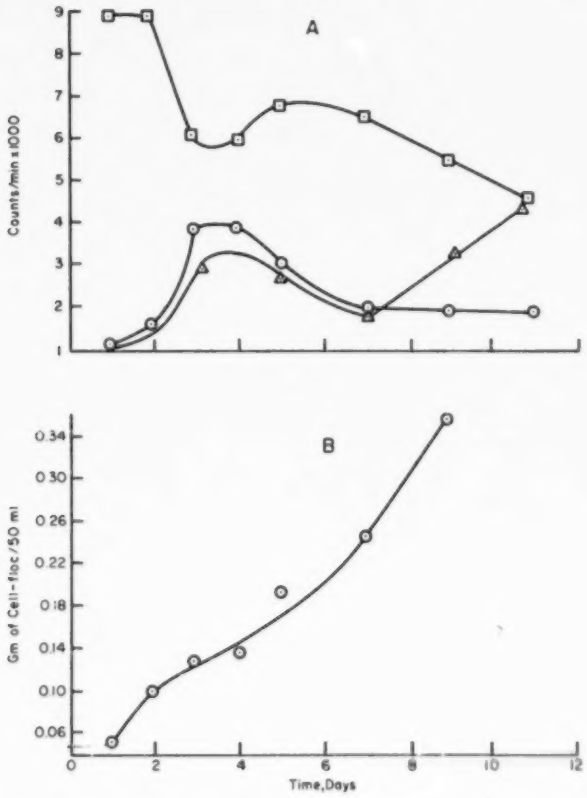

FIG. 3. Accumulation of Zn^{65} by *Zoogloea* 115 cell-floc during growth. a. Curve showing □ counts per minute per 50 ml of growth medium supernatant vs time, ⊙ counts per minute per 0.2 g of cell-floc vs time, △ counts per minute per total amount of cell-floc accumulated during growth period. b. Curve showing total weight of cell-floc accumulation per ml vs time.

in 3 ml distilled water, mixed by stirring, transferred to a counting vial, and recentrifuged. The supernatant was decanted; the cell paste was dried in an oven at a maximum of 180 C, then weighed and counted using an automatic gamma well counter (Nuclear-Chicago, Model 4216).

Figure 3a shows the accumulation of Zn^{65} in the presence of 10 μg carrier Zn/ml of growth medium by cells during an 11-day period. Loss of Zn^{65} from the culture medium is also shown. Loss of Zn from supernatant correlates with uptake of Zn by cells when total counts/min are compared. A plot of accumulation of Zn^{65}/0.2 g cells indicates that the rate of Zn^{65} uptake is constant from the 6th to the 11th day. Total uptake during this time increases because of the net increase in cell-floc weight. Total weight increase of cell-floc vs time is shown in Figure 3b. We emphasize weight of cell-floc because we believe cell numbers do not increase in proportion to weight over the entire 11-day period. The decrease in Zn associated with the cell-floc fraction between the 5th and 7th day suggests a shift in metabolism of the organism. This could reflect

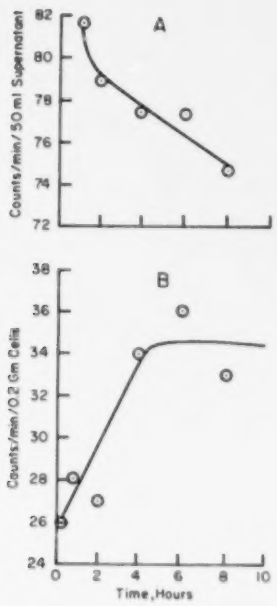

FIG. 4. Accumulation of Zn^{65} by 4-day-old culture of *Zoogloea* 115 cell-floc. a. Counts per min per 50 ml of growth medium supernatant (X 100). b. Counts per min per 0.2 g cells (dry wt) (X 100).

either a conversion from cell reproduction to zoogleal matrix synthesis or accumulation and utilization of poly-B-hydroxybutyrate within the cells. The release of Zn^{65} from cell-floc back into the growth medium during the 5-7-day interval appears to be real. Similar curves were obtained with Zn^{65} alone or with Zn^{65} plus 1 ppm carrier Zn, although these are not shown. Since we do not know the reason for this phenomenon, we decided to examine the uptake of Zn^{65} by cells when Zn was added during the growth cycle. For this purpose we added Zn^{65} after 1 day, 3 days, or 4 days growth, allowed the cells to continue growth, and then monitored the accumulation by the cells.

Again we found similar patterns of accumulation with Zn^{65} alone or with Zn plus carrier using the same age cells although the absolute values varied. The uptake curves were also similar with 1-day, 3-day, and 4-day cells. Figure 4 shows very rapid Zn^{65} accumulation (no carrier) by 4-day *Zoogloea* 115 cells.

Although the uptake curves on 1-day, 3-day, and 4-day cells are similar the absolute values varied, suggesting that the affinity of cells for metal ion varies with growth state or physiological state of the organism. For example, 1-day cells showed an average 1400 counts/min/0.2 g cells (dry wt) after 4 to 6 hours; 3-day cells had an average of 2750 counts/min/0.2 g cells after the same time. This indicates to us that actively growing cells (24-hour culture) have half the affinity for Zn accumulation of 4-day cells, in general agreement with data presented in Figure 3a.

We feel that the difference between 1-day and 4-day cells is a shift toward greater synthesis of zoogleal material, and we have been able to fractionate zoogleal matrix

from cell material and to demonstrate that Zn^{++} can be removed from solution by the zoogleal material. We have not determined the ratio of metal uptake by isolated zoogleal material in comparison to cells and this subject will be considered in a later communication.

General Discussion

The accumulation of metallic ions by microorganisms has been studied previously (e.g., Morgan, 1961), and is not unique with *Zoogloea*. Many chemical compounds of biological origin are known to complex and therefore concentrate metal ions from solution. *Zoogloea* strains have long been recognized as primary contributors to the successful function of aerobic waste treatment processes (Butterfield, 1935; Wattie, 1942) and therefore represent a potentially useful means for practical removal of ions from water. *Zoogloea* species of bacteria are floc formers in addition to their ability to produce a zoogleal matrix. The organisms settle out of suspension, affording a convenient means of separating ions from effluents once they have been complexed to the bacterial cell and/or gelatinous matrix. Strains of this organism are indigenous to a variety of aquatic ecosystems which suggests that one or another of the strains could function effectively under a variety of conditions. Although we have presented data obtained from only two isolates (115 and I-16-M), we have acquired several other strains from other investigators and find that each responds differently to nutritional and physical variables. Growth temperature is particularly critical for synthesis of the zoogleal matrix.

We wish to emphasize the potential utility of the organism for removal of radionuclide contaminants from water. In this regard, Polikarpov (1966) has discussed the extent to which the hydrosphere has been and is currently being contaminated by radionuclides. Although the only data presented in this paper pertaining to isotope accumulation is with Zn^{65}, the clear indication that Cu^{++}, Co^{++}, Ni^{++}, and Fe^{+3} are concentrated has relevance to uptake of isotopes of these ions.

Data presented using low concentrations of Zn^{++} in growing cultures undoubtedly is slightly biased because of incorporation of Zn^{++} as an enzyme cofactor within cells. This becomes a very minor consideration when carrier zinc reaches a 10 μg/ml concentration. Calculated coefficients of Zn^{++} accumulation is nearly the same when Zn^{65} is added without a carrier (151) as when 10 μg/ml Zn carrier is added (147).

All experiments have utilized batch culture techniques, but relationships to continuous flow systems should be determined. This is particularly important because ion uptake appears to be related to the physiological state of the organism. Such variables as C:N ratio and other nutritional factors undoubtedly have a direct bearing on the problem. Studies related to selective uptake of metal ions from mixtures are also to be conducted.

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